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Evaluation of Biofilm Formation by Three Different Methods and its Antibiogram with Special Reference to Indwelling Medical Devices from a Tertiary Care Hospital

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ABSTRACT

Backgound: Biofilms represent the exopolysaccharides produced by the bacteria on various indwelling devices in which they remain enmeshed. These bacteria are highly resistant to antimicrobial agents causing chronic and recurrent infections. So the present study was undertaken to detect biofilms from clinical isolates by three different methods with its antibiotic resistance pattern and association with various indwelling devices.

Material and Methods: The study was carried out in the Department of Microbiology, Indian Institute of Medical Science and Research Jalna over a period of three months .This is a cross-sectional type of observational study. A total of 112 clinical isolates were first identified by standard microbiological tests and then screened for biofilm formation by 1)Tube method,2)Tissue culture method and 3) Congo red agar method. Their antibiotic sensitivity pattern was determined using Kirby Bauer Disc Diffusion method and association with indwelling medical devices was observed prospectively& retrospectively.

Results: Out of the 112 clinical isolates,we found 57(50.9%) isolates produced biofilm by tissue culture method,33(29.46%) by tube method and only16(14.25%) by Congo red agar method.The predominant biofilm producer was Pseudomonas(70%) followed by Staphylococcus aureus(61.1%), Klebsiella (45.83%),Coagulase negative Staphylococcus(42.85%)and then E coli(32.14%).All the biofilm producing strains were highly resistant to commonly used antibiotics and there was a strong association between biofilm production and indwelling medical devices used for the diagnostic or therapeutic intervention.

Conclusions: Tissue culture method was the most sensitive method for detection of biofilms. As the resistance of these isolates was very high, all the isolates from the medical devices should be screened both for biofilm production and their antibiotic sensitivity testing should be performed for better patients compliance and outcome.

Keywords: *Biofilms, Enterococcus, Staphylococcus, Staphylococcus*

Introduction

Microorganisms have the potential to live in one of the two phenotypes: sessile or planktonic. Planktonic are free floating microorganisms. [1] Sessile phenotype results from attachment to solid surfaces, their irreversible binding and development of exopolysaccharide with gradual increase in the thickness of cellular exudates. Biofilms represent these cellular aggregates enmeshed within the exopolysaccharide matrix. [2]

Various indwelling medical devices like urinary catheter, endotracheal tubes, intravascular catheters, artificial joints etc., act as the nidus for the formation of these biofilms. In addition, microorganisms enmeshed in these biofilms evade antimicrobial challenges by multiple mechanisms. [3] These mechanisms include 1) Failure of an agent to penetrate the full depth of the film. 2) Some of the cells in a biofilm experience nutrient limitation and therefore exist in a slow growing or starved state making them not very susceptible to many antimicrobial agents.3) Some of the cells in a biofilms adopt a distinct and protected biofilm phenotype which is biologically programmed response to growth on a surface.

Biofilms are produced by both Gram-positive and Gram-negative bacteria. *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa* are commonly associated with the production of biofilms. [4]

The expression of biofilms in these bacteria is mainly determined by operons . Operons are the clusters of coregulated genes with related functions. The series of genes in an operon are transcribed as a single mRNA and consists of an upstream promoter and a downstream terminator. [5] The biofilm production in Staphylococcus spp is regulated by the expression of polysaccharide intracellular adhesion (PIA) which is the gene product of

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operon *icaADBC.* [6] In *Pseudomonas aeruginosa,* various operons that code for alginate, polysaccharides (*psl and pel*), proteins, cyclic-di-GMP-regulated adhesin A protein (*cdrA*), cup fimbria, type IV pili , lectins and extracellular DNA (eDNA) have been elucidated for the production of extracellular polysaccharide. [7]

With the advancement in medical fields, the use of indwelling medical devices is on the rise which further facilitates biofilm formation and chronicity of infection. There are various methods for biofilm detection which include Tissue culture plate method , Tube method, Congo red agar method, bioluminescent assay and light or fluorescent microscopic examination. The information of biofilm production would help the clinician to evaluate the measure of its virulence and then decide the line of treatment. [8,9] So the present study was undertaken to follow-up all the clinical isolates in our hospital for biofilm production , to assess their antibiotic sensitivity pattern and to find out their association with indwelling medical devices.

Materials and methods

Setting and Design: The study was carried out in the Department of Microbiology of Indian Institute of Medical science and Research ,Warudi, Jalna for a period of three months from July 2016 to September 2016

Inclusion Criteria: After obtaining clearance from the ethical committee of the Institiute ,all the patients admitted to the hospital were included in the study .The samples that were sent to microbiology department included pus, sputum, urine ,blood ,body fluids like pleural fluid, ascitic fluid etc.The isolates were identified by standard microbiological techniques (Gram stain,motility,culture on blood agar, MacConkeys agar, and biochemical identification). [10,11]

The samples that showed positive growth were then analysed prospectively and retrospectively for the use of any indwelling medical device in each of these patients.

Bacterial Strains: A total of 112 clinical isolates were subjected to biofilm detection methods. As a positive biofilm producer,we used *Staphylococcus epidermidis* ATCC 35984 and *Staphylococcus aureus* ATCC 35556, as the reference strain. *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218 and *Staphylococcus epidermidis* ATCC 12228 (non-slime producer) were used as control.

Detection of Biofilm Formation: All the isolates were subjected to biofilm production by the three methods.

Tube Method (TM): A qualitative assessment of biofilm formation was done, as previously proposed by Christensen *et al.* [9] A loopful of microorganism from overnight culture plates were inoculated in Trypticase Soya Broth with glucose (10mL) and incubated for 24 hours at 37°C. The tubes were decanted and washed with Phosphate Buffer Saline (pH 7.3) and dried.They were then stained with crystal violet (0.1%). Excess stain was removed and they were then washed with deionized water. Tubes were again dried in inverted position and observed for biofilm formation.

When a visible film lined the wall and bottom of the tube, biofilm formation was considered positive. If there was a ring formation at the liquid interface, it was not indicative of biofilm formation. The tubes were examined for biofilm formation and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong. The experiment was done in triplicate and repeated thrice.

Congo Red Agar Method.(CRA) A simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium was described by Freeman et al. [12] CRA medium was prepared using brain heart infusion broth 37 g/L, sucrose 50 g/L, agar No. 1, 10 g/L and Congo Red indicator 8 g/L. First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121ºC for 15 minutes) separately from the other medium constituents and it was then added to the autoclaved brain heart infusion agar with sucrose at 55ºC.[13] Test organisms were inoculated on Congo Red Agar plates and incubated at 37ºC for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production. The experiment was performed in triplicate and repeated three times.

Tissue Culture Plate (TCP) Method: Overnight broth cultures of bacteria in Trypticase soya broth (TSB) with 1% glucose were diluted 1 : 100 . 200 µl portions were inoculated in 96 well flat bottom polystyrene microtitre plates (Nuclon TM Ltd). [14] Microtitre plates were incubated at 35 ºC for 24 hours. Cultures were then aspirated from these wells and the wells were washed 3 times with phosphate buffer saline at pH 7.2. The plates were then air dried overnight and stained with 0.1% crystal violet.The optical density of the wells was measured at 490 nm using micro ELISA auto reader. (Robonic- Readwell touch –Automatic ELISA plate analyser).An optical density of 0.12 was considered as positive to distinguish biofilm producers from those that did not form biofilm. Sterile TSB was used as a negative control (Blank). Intensity of Biofilm was classified as given by Mathur et al. [6] (Table 1)

Antibiotic Sensitivity Testing: Antibiotic sensitivity testing was performed as per CLSI guidelines [15] using Kirby Bauer's Disc diffusion method on Mueller Hinton

Agar plate with the following antibiotics. Ofloxacin(5µg), Cefoxitin (30µg), Cefotazidime & Clavulonic acid (30/10µg), Erythromycin (15µg), Cefipime (30µg), Cotrimoxazole (25µg), Amikacin (30µg), Gentamicin (10µg), Nitrofurantoin (30µg), Linezolid (30µg), Vancomycin (30µg), Imipenem (10µg), Pipercillin &Tazobactum (100/10µg), and Colistin (10µg). Antibiotics discs were procured from HiMedia Laboratories Pvt. Ltd, India.

Results

Out of 112 isolates , tissue culture plate showed the maximum 57 (50.9%) isolates positive for biofilm production, of which 20(17.85%) showed high biofilm production and 37(33.04%) showed moderate biofilm production. (Table 2).Tube method showed biofilm production in 33 (29.46%) isolates among which 12(10.71%) showed high biofilm production and 21(18.75%) showed moderate biofilm production. Biofilm production in Congo red agar method was seen in only 16 (14.28%) of isolates.

On statistical analysis, the sensitivity and positive predictive value of tube method was more than that of Congo red agar method although the specificity of both remained the same. (Table 3)

Of all the isolates, *Pseudomonas aeruginosa* showed maximum biofilm production 70%, followed by *Staphylococcus aureus* (59.46%), Klebsiella spp (44%), *Staphylococcus epidermidis* (42.85%), Lastly *E coli* showed biofilm production in only (32.14%) of isolates. (Table 4).

Maximum association between biofilm production and indwelling medical devices was observed with *Staphylococcus aureus* (40%)

When the resistance pattern of biofilm producing isolates was seen, it was found that in Gram positive bacteria,72.73% of *Staphylococcus aureus*, and 66.66% of Coagulase negative Staphylococcus were resistant to Erythromycin. (Table 5) Similar pattern of high resistance was observed to Cotrimoxazole (63.64% in *Staphylococcus aureus* and 100% in Coagulase negative Staphylococcus) Cefoxitin is considered as the surrogate marker for Methicillin resistant Staphylococcus[15].In our study more than 60% of the strains were found to be Methicillin resistant(as they were resistant to Cefoxitin).

In gram negative bacteria , *Pseudomonas aeruginosa* isolated from the indwelling devices showed a very high resistance to Amikacin (71.43%) and Cefipime. Of the nine strains of *E coli* that were biofilm producing, (71.43%) were resistant to Cefipime and 6 strains (66.67%)were resistant to Gentamicin, Ceftazidime& Clavulonic acid, and Cotrimoxazole.

Table 1: Classification of bacterial adherence by TCP method.

Mean OD values	Adherence	Biofilm formation
<0.120	Non	Non / weak
0.120-0.240	Moderately	Moderate
>0.240	Strong	High

Table 2: Bio film production in the clinical isolate by each of the methods.

Bio film formation	TCP	TM	CRA
High	20 (17.85%)	12 (10.71%)	05 (4.46%)
Moderate	37 (33.04%)	21 (18.75%)	11 (9.82%)
Weak / none	55 (49.11%)	79 (70.54%)	96 (85.71%)

Table 3: Statistical evaluation of TM and CRA methods for detection of bio film formation in clinical isolates (n=112) taking tissue culture as the gold standard.

Screening methods	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Tube method	57.89%	92.73%	89.19%	68.00%
Congo red agar method	28.07%	92.73%	80%	55.43%

Table 4: Correlation of bio film production with organisms isolated (n=112).

Organisms	No. Of isolates n=112	Bio film production in the isolate by either of the methods
Pseudomonas aeruginosa	10	7(70%)
Stapylococcus. aureus	37	22(59.46%)

Organisms	No. Of isolates n=112	Bio film production in the isolate by either of the methods
Klebsiella spp	25	11(44%)
Staphylococcus epidermidis	7	3(42.85%)
E. coli	28	9(32.14%)
Acinetobacter spp	2	0
Enterococcus spp	1	0
Candida species	2	0

Table 5: Resistance pattern of the bio film producing isolates.

Antibiotics	Gram positive		Antibiotics	Gram negative		
	Staphylococcus aureus n=22(%)	CONS n=3(%)		E. coli n=9(%)	Klebsiella spp n=11(%)	Pseudomonas aeruginosa n=7 (%)
Erythromycin	16(72.73)	2(66.67)	Gentamicin	6(66.67)	1(9.09)	2(28.57)
Cotrimoxazole	14(63.64)	3(100.00)	Amikacin	2(22.22)	2(18.18)	5(71.43)
Cefoxitin	14(63.64)	2(66.67)	Cefepime	7(77.78)	5(45.45)	5(71.43)
Gentamicin	9(40.91)	1(33.33)	Ceftazidime+ Clavulonic acid	6(66.67)	2(18.18)	0.00
Linezolid	0.00	0.00	Ofloxacin	0.00	0.00	1(14.29)
Clindamycin	5(22.73)	0.00	Co-trimoxazole	6(66.67)	4(36.36)	Not tested
Nitrofurantoin	0.00	1(33.33)	Imipenem	Not tested	Not tested	1(14.29)
Vancomycin	0.00	0.00	Piperacillin + Tazobactum	Not tested	Not tested	3(42.86)
			Colistin	Not tested	Not tested	3(42.86)

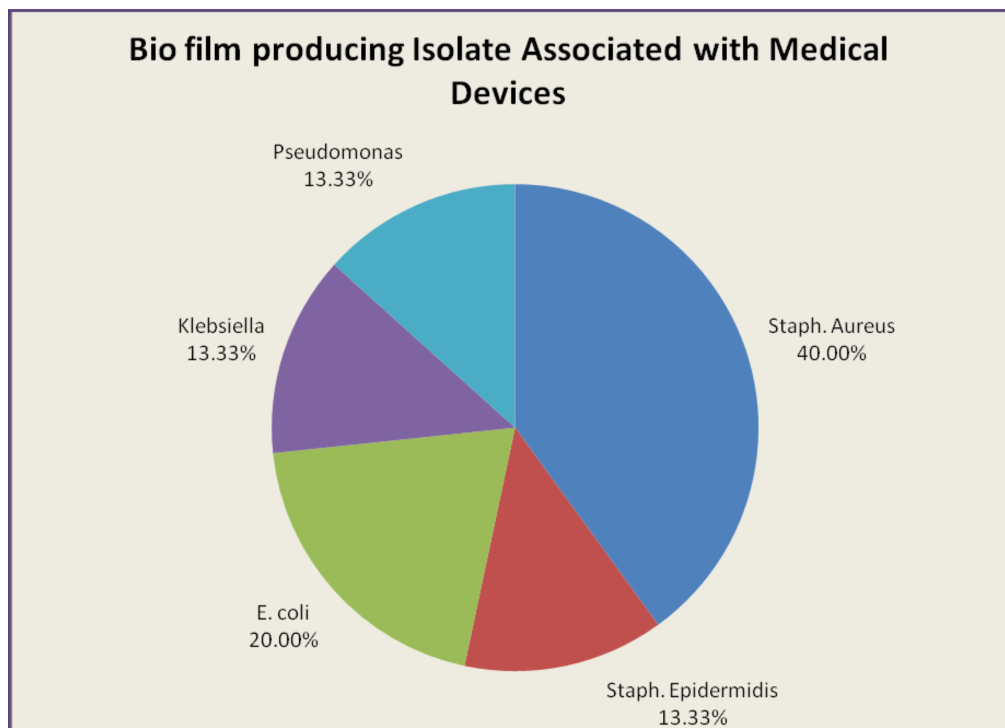

Fig. 1: Correlation of bio film producing organisms isolated and medical devices.

Discussion

Biofilms represent an important defense mechanism for microorganisms on indwelling medical devices which enables them to evade the antibiotics so that they can survive for longer period of time .We screened a total of 112 isolates from our hospital for biofilm production by either of the three methods i.e. Tissue culture plate method. Tube method ,Congo red agar method.

Tissue culture plate showed biofilm production in 50.9% of isolates of which strong biofilm production was found in 17.85% of isolates and moderate biofilm production was seen in 33.05% of isolates. Mathur et al [6] have reported strong biofilm production in 14.4% of isolates and moderate biofilm production in 39.4% while Nabajit deka [17] have reported strong biofilm production in 36% and moderate biofilm production in 47% of isolates. The production of biofilm in our isolates was less compared to these findings which could be because of either of the two reasons ,firstly the incubation period we used, for this method was 18 hrs and secondly the absorbance we had used for ELISA was 490 mm. The biofilm production is enhanced in presence of glucose and if the incubation period was increased further. [6] Tube method correlated best with the findings of Tissue culture plate method. Of the 29.46%of biofilm producing isolates ,10.71% showed high biofilm production and 18.75% showed moderate biofilm production but this was again subjected to interobserver variation. Bose et al [18] have reported biofilm production by tube method in 42.46% of isolates while Mathur et al [6] have reported biofilm production in 41 % of isolates respectively. Congo red agar method was found to be the least sensitive with only 14.28% of isolates showing biofilm formation .Only 5 (4.46%) isolates produced black coloured colonies while the rest 11 (9.82%) showed pink coloured colonies with crystalline consistency so they were considered as moderately positive. Hasan et al [19] have reported biofilm production by Congo red in 9.9% of isolates and Nabajit deka [17] in 20% of isolates. Our findings lie in between the two.

Statistically we found that if tissue culture method was used as a gold standard, Tube method had its sensitivity and positive predictive value more than Congo red agar method which corresponded with the findings of Mathur et al. [6] Most of the *Pseudomonas aeruginosa* isolates (70 %) produced biofilms and the resistance they showed to antibiotics varied from 14% (Ofloxacin and Imipenem) to 71.43 % for (Cefipime & Gentamicin) Hyderi et al [20] have reported 43.5 %of *Pseudomonas aeuginosa* producing biofilms in burns patients also Perez at al [21] have done a study on cystic fibrosis patients and found that 71.4% of cystic fibrosis patients produced biofilms. Staphylococcus species was next isolate which produced maximum biofilms. We found 59.46% of *Staphylococcus aureus* strains and 42.85% of Coagulase negative strains producing biofilms in our study. Sharvari et al [22] reported 45.6% of *Staphylococcus aureus* and 38.7% of Coagulase negative Staphylocoocus producing biofilms. Also Mathur et al [6] and Taj et al [8] have reported biofilms in 57.8%and 54.78% of Staphylococcus isolates. Of all the Staphylococcus isolates that produced biofilms,40% were associated with indwelling medical devices . Kloos et al [23] have reported Staphylococcus as the predominant species isolated on polymeric devices , their major pathogenic factor being ability to form biofilms on these surfaces. 44% of Klebsiella spp strains produced biofilms by either of the methods .Seifi et al [24] have shown fully established biofilm production in 33% of *Klebsiella pneumonia* and moderate biofilm production in 52.1% strains of *Klebsiella pneumonia*.

32.14 %of *E coli* strains in our study revealed biofilm production while the study done by Suman et al [25] had revealed 92% of E coli producing biofilms in uropathogens. Our incidence is less because we had isolated *E coli* from all possible samples and not restricted ourselves to urinary isolates.

Almost all the isolates showed resistance to antibiotics implying the chronic nature of these infections. Our findings of drug resistance corresponded with that of Sundaram et al [26]

Conclusion

Tube method can be used as an important screening method for detection of biofilms in resource poor settings. If facilities are available, then tissue culture method can be the method of choice. As biofilm producing strains isolated from indwelling medical devices are resistant to commonly used antibiotics, all such biofilm producing strains should be subjected to antibiotic sensitivity testing for better patient management and compliance.

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